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Supplemental Information

TMEM150C/Tentonin3 Is a Regulator of Mechano-gated Ion Channels

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Supplemental data

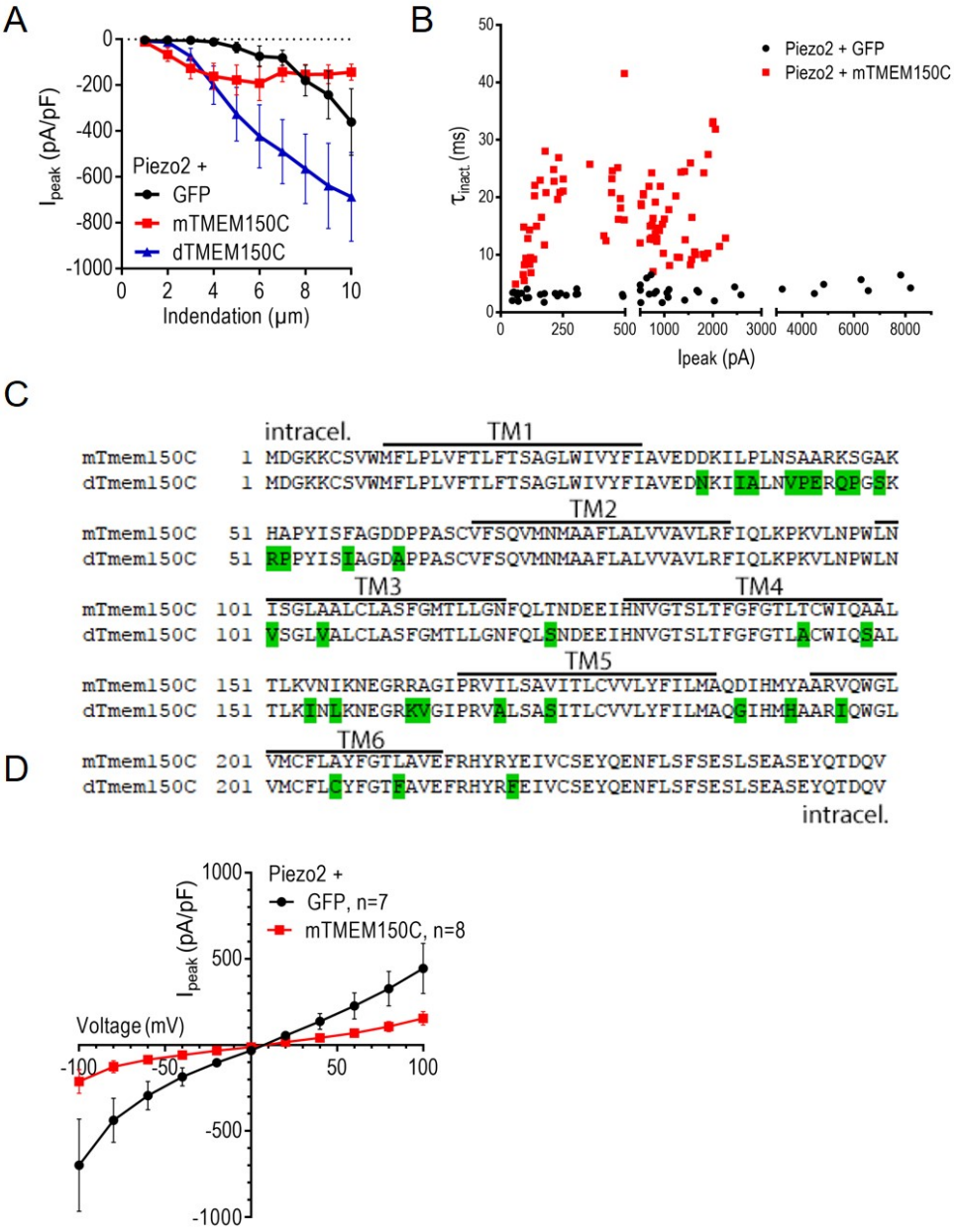

Figure S1. Related to Figure 1.

(A) Peak whole-cell MA current density measured in HEK293T$^{\Delta P1}$ cells expressing Piezo2 with GFP or with mouse or duck TMEM150C (E_{hold} = -80 mV) in response to mechanical indentations with a glass probe.

(B) TMEM150C prolongs Piezo2 MA current inactivation rate independently of peak current amplitude. MA current inactivation rate measured at different peak MA current amplitudes in HEK293T$^{\Delta P1}$ cells expressing Piezo2 with GFP or with mouse TMEM150C (E_{hold} = -80 mV).

(C) Amino acid alignment of mouse TMEM150C (mTMEM150C, NP_878261.1) and duck TMEM150C (dTMEM150C, MG697237). The proteins share 87% amino acid identity. Putative transmembrane domains are denoted by black bars.

(D) Peak MA current density-voltage plots measured in HEK293T$^{\Delta P1}$ cells in response to mechanical indentation of 5-10 µm for Piezo2+GFP and 4-9 µm for Piezo2+mTMEM150C.

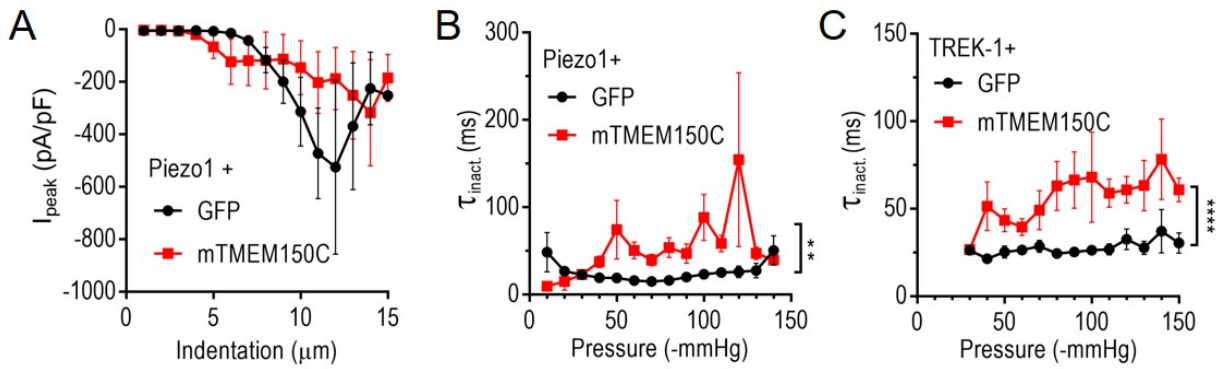

Figure S2. Related to Figures 2, 3 and 4.

(A) Peak whole-cell MA current density measured in HEK293T$^{\Delta P1}$ cells expressing indicated constructs, in response to mechanical indentation with a glass probe at E_{hold} = -80 mV.

(B and C) TMEM150C prolongs the rate of MA current inactivation in HEK293T$^{\Delta P1}$ cells expressing Piezo1 (*B*) or TREK-1 (*C*). MA currents were elicited in the cell-attached mode by high-speed pressure clamp from a holding potential of -60 mV (Piezo1) or 0 mV (TREK-1). $^{**}P < 0.01$, $^{****}P < 0.0001$ for expression construct effect, two-way ANOVA.

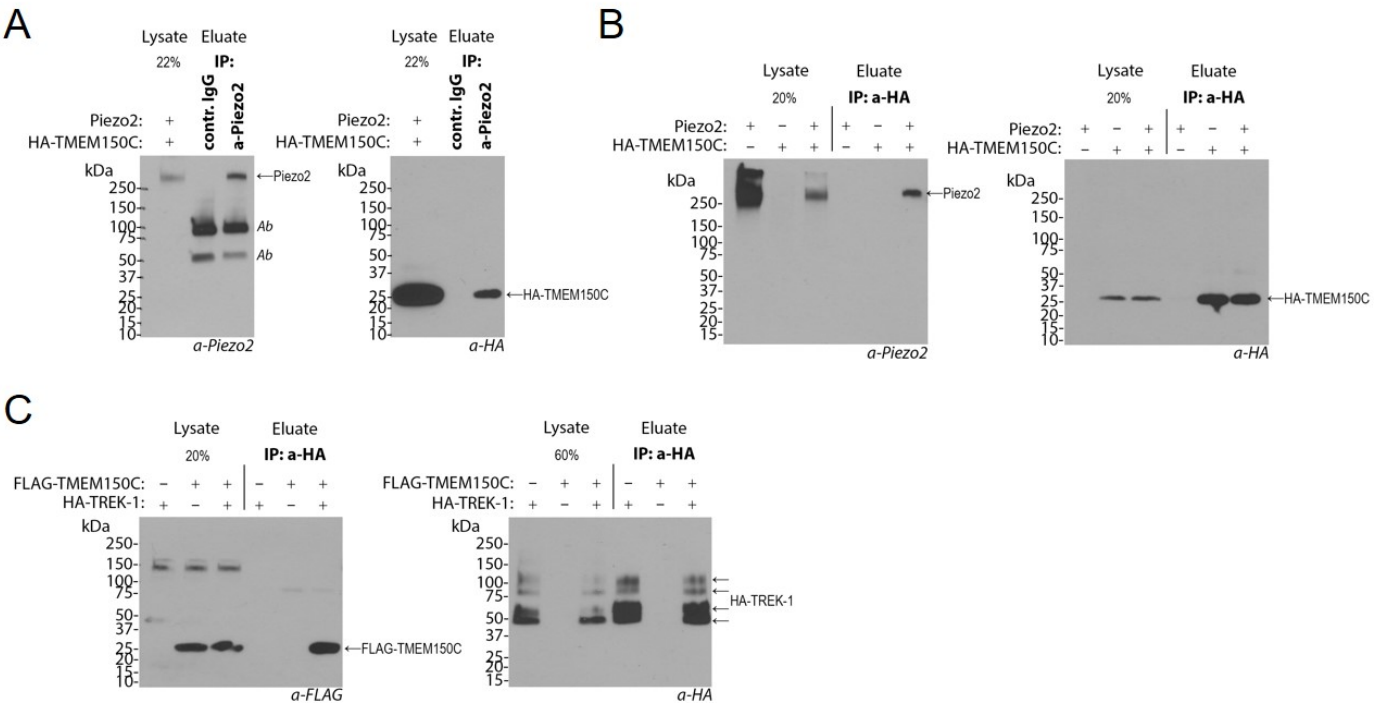

Figure S3. Related to Figures 1 and 4.

TMEM150C co-immunoprecipitates with Piezo2 and TREK-1 from HEK293T$^{\Delta P1}$ lysates.

(A–C) Co-immunoprecipitation and immunoblot analysis of the indicated mouse proteins from detergent lysates of HEK293T$^{\Delta P1}$ cells using antibodies immobilized on magnetic beads. Equal initial amounts of lysates were used for all immunoprecipitations. Percentages indicate the amount of lysate loaded on the gel relative to eluates. Calculated molecular weights for single subunits: Piezo2 (326 kDa), HA-TMEM150C (29 kDa), FLAG-TMEM150C (29 kDa), HA-TREK-1 (46 kDa). HA-TREK-1 migrates as four bands (bottom to top): monomer, glycosylated monomer, dimer, glycosylated dimer. *Ab*, antibody fragment from immunoaffinity beads.

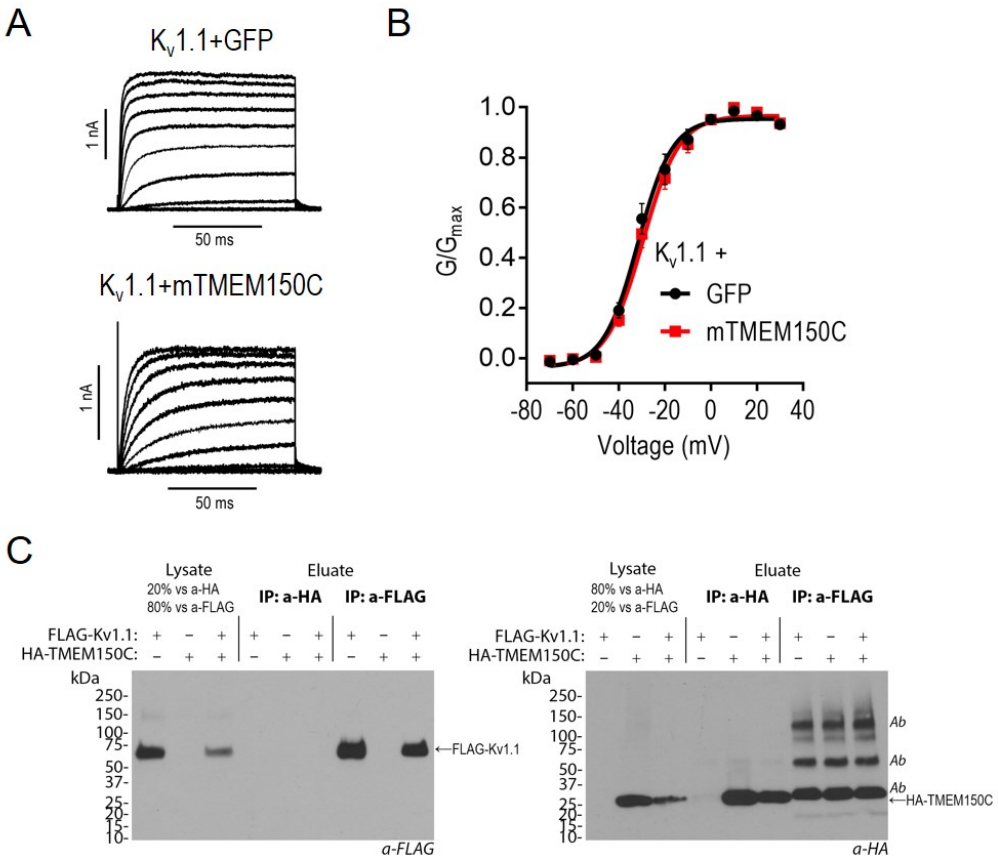

Figure S4. Related to Figure 4.

TMEM150C does not affect voltage dependence of activation of K_v1.1.

(A) Exemplar whole-cell current families recorded from mouse K_v1.1 in HEK293T$^{\Delta P1}$ in the presence or absence of mouse TMEM150C in response to a voltage step protocol from -70 mV to 30 mV in 10 mV increments, from a holding potential of -70 mV.

(B) Normalized conductance from current families recorded from K_v1.1 fit with a Boltzmann function. $V_{1/2}$ = -31.7 ± 0.9 mV (K_v1.1 + GFP, n = 13), -29.6 ± 0.8 mV (K_v1.1 + mTMEM150C, n = 11).

(C) TMEM150C does not co-immunoprecipitate with K_v1.1 from HEK293T$^{\Delta P1}$ lysates. Equal initial amounts of lysates were used for all immunoprecipitations. Percentages indicate the amount of lysate loaded on the gel relative to eluates. Calculated molecular weights for single subunits: HA-TMEM150C (29 kDa), FLAG-K_v1.1 (60 kDa). *Ab*, antibody fragment from immunoaffinity beads.

Supplemental Experimental Procedures

Resource table

REAGENT OR RESOURCE	SOURCE	IDENTIFIER
Biological samples		
Trigeminal ganglia from adult domestic duck (Mallard)	This paper	
Deposited Data		
Duck (Mallard) TMEM150C	This paper	MG697237
Experimental Models: Organisms		
Domestic duck (Mallard): *Anas platyrhynchos domesticus*	MarWin Farm (New Hartford, CT)	
Experimental Models: cell lines		
HEK293T$^{PIEZO1-/-}$ (HEK293T$^{\Delta P1}$)	Dr. Ardem Patapoutian (Scripps Research Institute) (Lukacs et al., 2015)	
Oligonucleotides		
Mouse TMEM150C cloning FWD GGCATGGACGGGAAGAAATGC	This paper	
Mouse TMEM150C cloning Rev CCAAGGACAAACTGTTGCTACACC	This paper	
Duck TMEM150C cloning and *in situ* probe FWD GGTATGGACGGGAAGAAATGC	This paper	
Duck TMEM150C cloning and *in situ* probe Rev GGCTACACCTGATCTGTCTGG	This paper	
Recombinant DNA		
Mouse-Piezo2-Sport6	Dr. Ardem Patapoutian (Scripps Research Institute) (Coste et al., 2010)	ADN28065.1
Mouse-Piezo1-pMO	This paper	ADN28064.1
Mouse-TMEM150C-IRES2-GFP	This paper	NP_878261.1
Mouse-HA-TMEM150C-IRES2-GFP	This paper	
Mouse-FLAG-TMEM150C-IRES2-GFP	This paper	
Duck-TMEM150C-IRES2-GFP	This paper	MG697237
Mouse-TREK-1-pMO	This paper	NP_034737.2
Mouse-HA-TREK-1-pMO	This paper	
Mouse- K_v1.1- Myc-FLAG-pCMV6	OriGene	MR222106
IRES2-GFP	Clontech	
Antibodies		

Anti-HA tag (mouse)	Sigma-Aldrich	clone HA7 #H3663
Anti-FLAG tag (rabbit)	Sigma-Aldrich	F7425
Anti-Piezo2 (rabbit)	(Schneider et al., 2017)	57-1
Secondary anti-mouse-HRP	ThermoFisher	31430
Secondary anti-rabbit-HRP	ThermoFisher	31458
Software and Algorithms		
Prism 7.01	Graph pad	
pCLAMP 10	Molecular Devices	
MATLAB R2014b	MathWorks	
IGOR Pro 6.37	WaveMetrics	

Contact for Reagent and Resource Sharing

Further information and requests for resources and reagents should be directed to Sviatoslav Bagriantsev (slav.bagriantsev@yale.edu).

Supplemental experimental procedures

RNA *in situ* hybridization. Trigeminal ganglia from adult birds were processed and developed with alkaline phosphatase-conjugated anti-digoxigenin Fab fragments as described previously (Schneider et al., 2014). Dissected TGs were fixed in 4% paraformaldehyde in phosphate-buffered saline for 2 hrs at 4ºC, sectioned at 12-15 µm thickness and probed with digoxigenin-labeled cRNA generated using T7/T3 *in vitro* transcription from transcript fragments amplified from duck TG cDNA (see Resource Table for primers). Signal was developed with alkaline phosphatase-conjugated anti-digoxigenin Fab fragments.

Molecular cloning. Standard cloning techniques were used, and all plasmids were verified by full-length sequencing. Mouse TREK-1 ($K_{2P}2.1$) with and without the human influenza hemagglutinin (HA) tag on the N-terminus was cloned from the pGEMHE vector (Bagriantsev et al., 2012) into the pMO vector for expression in mammalian cells. Mouse TMEM150C was cloned from dorsal root ganglia into the IRES2-GFP vector. Mouse TMEM150C sequence used here is identical to that published elsewhere (GenBank accession number NP_878261.1)(Hong et al., 2016). Tagged versions of TMEM150C were made by adding the HA (YPYDVPDYA) or FLAG (DYKDDDDK) sequence to the N-terminus. Duck TMEM150C was cloned from adult duck trigeminal ganglia into IRES2-GFP. The sequence of duck TMEM150C was deposited to GenBank under the accession number (MG697237):

MDGKKCSVWMFLPLVFTLFTSAGLWIVYFIAVEDNKIIALNVPERQPGSKRPPYISIAGDAPPASCVFSQVMNMAAF
LALVVAVLRFIQLKPKVLNPWLNVSGLVALCLASFGMTLLGNFQLSNDEEIHNVGTSLTFGFGTLACWIQSALTLKI
NLKNEGRKVGIPRVALSASITLCVVLYFILMAQGIHMHAARIQWGLVMCFLCYFGTFAVEFRHYRFEIVCSEYQENF
LSFSESLSEASEYQTDQV

Cell Culture. HEK293T$^{\Delta P1}$ cells were cultured in DMEM with 10% FBS, 1% Penicillin/Streptomycin, and 2 mM glutamine. Cells prepared for Piezo1 cell-attached recordings were transfected using Lipofectamine3000 (ThermoFisher) following manufacturer's instructions. For all other experiments, cells were co-transfected using Lipofecatmine2000 (ThermoFisher) according to the manufacturer's instructions.

Co-transfections were 2 µg of total plasmid at a mass ratio of 1:1 except for the following: 1.5 µg Mouse-Piezo2-Sport6 + 0.5 µg IRES2-GFP (for whole-cell); 1.5 µg Mouse-Piezo2-Sport6 + 0.5 µg Mouse-TMEM150C-IRES2-GFP (for whole-cell); 1.5 µg Mouse-Piezo2-Sport6 + 0.5 µg IRES2-GFP (for cell-attached); 0.5 µg TREK-1 + 1 µg IRES2-GFP + 0.5 µg pMO (for cell-attached); 0.5 µg TREK-1 + 1 µg Mouse-TMEM150C-IRES2-GFP + 0.5µg pMO (for cell-attached).

Immunoprecipitation. Cellular lysates were obtained by nutating HEK293$^{\Delta P1}$ cells for 20 min at 4ºC in Lysis Buffer (100 mM NaCl, 20 mM KCl, 20% (v/v) glycerol, 5 mM EGTA, 1% CHAPS (w/v), 10 mM HEPES/NaOH pH 7.4) supplemented with antiproteases (Sigma-Aldrich #11697498001) and cleared by centrifugation at 14,000 x g for 10 min at 4ºC. For anti-HA and anti-FLAG tag pull downs, lysates (200 µg of total protein in 500 µl) were incubated with anti-HA or anti-FLAG magnetic beads for 1 hr at room temperature, following the manufacturer's instructions (Pierce #88838 for HA beads, Pierce #A36797 for FLAG beads). For anti-Piezo2 pull downs, lysates were incubated overnight at 4ºC with polyclonal rabbit antibody against Piezo2 (#57-1) (Schneider et al., 2017) or control rabbit IgG (Sigma-Aldrich #I5006) covalently linked to magnetic beads, following the manufacturer's instructions (Pierce #88805). Beads were washed three times with 800 µl of Lysis Buffer, eluted in 100 µl of pre-heated to 95ºC non-reducing Laemmli buffer with 1% SDS, and analyzed by Western blotting on a PVDF membrane using monoclonal mouse antibodies against the HA tag (Sigma-Aldrich #H3663), rabbit polyclonal antibodies against the FLAG tag (Sigma-Aldrich #F7425), or polyclonal rabbit antibodies against Piezo2 (#57-1) (Schneider et al., 2017). Signals from horseradish peroxidase-conjugated secondary antibodies were developed using the SuperSignal chemiluminescent substrate (ThermoFisher #34050) and detected on an X-ray film.

Electrophysiology. Transfected HEK293T$^{\Delta P1}$ were plated onto matrigel-coated coverslips (BD Bioscience, Billerica MA, diluted 1:100 in PBS) the day following transfection (18-24 hrs). Cells were visualized using an Olympus BX51-WI with an Orca flash2.8 camera (Hamamatsu). Data were acquired using a Multi-clamp 700-B patch-clamp amplifier, Digidata 1500 digitizer (Molecular Devices) and pCLAMP software. Currents were low-pass filtered at 10 kHz. Recordings were not corrected for liquid junction potential.

For whole-cell recordings of mechano-activated currents from Piezo2 and Peizo1, data were collected at a sampling rate of 20 kHz using a 500 MΩ feedback resistor, as previously described (Schneider et al., 2017). Patch pipettes were made from 1.5 mm outer diameter borosilicate glass (Warner Instruments #G150F-3) using a P-1000 puller, and were fire polished to 1-4 MΩ tip resistance. Series resistance and membrane capacitance were compensated at 85%. Internal solution contained (mM): 133 CsCl, 5 EGTA, 1 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, 4 Mg-ATP, 0.4 Na_2-GTP, pH to 7.3 with CsOH. External solution contained (mM): 140 NaCl, 5 KCl, 10 HEPES, 2.5 $CaCl_2$, 1 $MgCl_2$, 10 glucose (pH 7.4 with NaOH). Cells were mechanically stimulated with a fire-polished, blunt glass probe (tip diameter ~2-4 µm) which was controlled by a pre-loaded Piezo actuator stack (Physik Instrumente Gmbh, DE) set at an angle of 32˚ from the horizontal plane. After break-in, the glass probe was positioned at the surface of the cell, just before cell displacement of the cell membrane would be observed. Cells were stimulated in 1 µm, 150 ms steps with a velocity of 1000 µm/s with 5 s between sweeps. All data were acquired within 5 minutes of break-in.

For whole-cell recording of voltage activation of $K_v1.1$, internal solution contained (mM): 150 KCl, 3 $MgCl_2$, 5 EGTA, 10 Hepes, pH to 7.2 with KOH. External solution was identical to that described above. Cells were held at -70 mV for 50 ms and briefly stepped to -80 mV for 20 ms to assess cell parameters. Then they were held for 50 ms at -70 mV before being subjected to 100 ms voltage stimulation steps, ranging from -70 mV to 30 mV in 10 mV increments. Following stimulation, cells were returned to -70 mV before the next sweep. Leak current was subtracted online using the P/4 method on pCLAMP. Series resistance and membrane capacitance were compensated at 85%. Analyzed and representative traces are an average of 3 protocols. Currents were averaged over 88-98 ms of each voltage step and converted to conductance using the equation $G = I / (V_m - E_K)$, where G is the conductance, I is the averaged current, V_m is the membrane potential and E_K is the reversal potential for potassium calculated for the solutions used at -86.16 mV. The conductance data were normalized by the maximum conductance data and fit with the Boltzmann equation, $G = G_{min} + (G_{max} - G_{min}) / (1 + exp\text{^}([V_{1/2} - V_m]/k))$, where G_{min} and G_{max} are minimal and maximal conductance, respectively, V_m is the voltage, $V_{1/2}$ is the voltage at which the channels reached 50% of their maximal conductance, and k is the slope of the curve.

For cell-attached recordings of mechanically activated current by high-speed pressure clamp (HSPC), data was collected at a sampling rate of 10 kHz using a 5 GΩ feedback resistor. Patch pipettes were made similarly to whole-cell pipettes, but with a tip resistance of 0.7-2 MΩ. Pipette solution contained (mM): 130 NaCl, 5 KCl, 10 HEPES, 10 TEA-Cl, 1 $CaCl_2$, 1 $MgCl_2$, pH to 7.3 with NaOH. Bath solution contained (mM): 140 KCl, 10 HEPES, 1 $MgCl_2$, 10 glucose, pH to 7.3 using KOH. Pressure control for seal formation and pressure stimulation protocols were controlled using a HSPC-1 high speed pressure clamp system (ALA Scientific Instruments). Cells were approached with a 10-20 mmHg positive pressure, and pressure was released to form a gigaseal. Following formation of the gigaseal, holding potential was set to -60 mV for Piezo1 recordings or 0 mV for TREK-1 recordings. Cells were subjected to stepwise, 200 ms negative pressure steps (Δ10 mmHg) preceded by a 500 ms pre-pulse at 5 mmHg to remove inactivation (Lewis and Grandl, 2015), with 3 seconds between stimuli.

Inactivation kinetics of mechano-evoked currents were determined by fitting current decay to a single-exponential decay function, as previously described (Schneider et al., 2017). Briefly, MA currents were fit to the following single-exponential decay equation: $I=\Delta I*exp\wedge(-t/\tau_{inact})$, where ΔI is the difference between peak MA current and baseline, t is the time from the peak current (the start of the fit), and τ_{inact} is the decay constant. Summary τ_{inact} from figures represent averages from traces with the top 75% of MA current, as previously quantified (Coste et al., 2010), unless described otherwise.

Post-stimulus whole-cell mechano current was quantified for each trace as the average of 20 ms of data 5 ms following the removal of stimulus. For cell-attached recordings, post-stimulus current was quantified for each trace as the average of 20 ms of data 150 ms after the removal of pressure stimulus, allowing for pressure readings to return to zero. Reported I_{peak}-normalized post-stimulus current is an average of traces which yielded 75% or more maximal peak current.

The apparent threshold of mechano activated current was determined as the first indentation to elicit a peak current greater than background noise, typically at least 40 pA above averaged baseline. Similarly, the apparent threshold of mechano activated current through HSPC stimulation was also the first pressure stimulation to elicit a peak current resolvable above background noise, typically at least 10 pA.

Statistical Analysis. Data were analyzed using a combination of Igor Pro 6.3 (Wavemetrics, converted from pCLAMP using TaroTools), Matlab (Mathworks), and GraphPad Prism 7.01 (GraphPad Software, Inc). Sample size and statistical tests are reported in figure legends. Statistical tests were chosen based on normality of distributions and variance equality, or lack thereof, and the number of samples. Data were reported as mean ± SEM, significance displayed as not significant (NS), $P > 0.05$, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, $****P < 0.0001$.